

1 **Revisiting rustrela virus – new cases of encephalitis and a solution to the**  
2 **capsid enigma**

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25 **Running Title:** Revisiting rustrela virus

26 **Key Words:** rustrela virus; rubivirus; sequencing; capsid; intergenic region; encephalitis;  
27 Eurasian otter; South American coati; yellow-necked field mouse.

## 28 **Abstract**

29 Rustrela virus (RusV, species *Rubivirus strelense*) is a recently discovered relative of  
30 rubella virus (RuV) that has been detected in cases of encephalitis across a wide spectrum  
31 of mammals, including placental and marsupial animals. Here we diagnosed two additional  
32 cases of fatal RusV-associated meningoencephalitis in a South American coati (*Nasua*  
33 *nasua*) and a Eurasian otter (*Lutra lutra*) that were detected in a zoological garden with  
34 history of prior RusV infections. Both animals showed abnormal movement or unusual  
35 behaviour and their brains tested positive for RusV using specific RT-qPCR and RNA *in situ*  
36 hybridization. As previous sequencing of RusV proved to be very challenging, we employed  
37 a sophisticated target-specific capture enrichment with specifically designed RNA baits to  
38 generate complete RusV genome sequences from both detected encephalitic animals and  
39 apparently healthy wild yellow-necked field mice (*Apodemus flavicollis*). Furthermore, the  
40 technique was used to revise three previously published RusV genomes from two  
41 encephalitic animals and a wild yellow-necked field mouse. Virus-to-host sequence ratio  
42 and thereby sequence coverage improved markedly using the enrichment method as  
43 compared to standard procedures. When comparing the newly generated RusV sequences  
44 to the previously published RusV genomes, we identified a previously undetected stretch  
45 of 309 nucleotides predicted to represent the intergenic region and the sequence encoding  
46 the N-terminus of the capsid protein. This indicated that the original RusV sequence was  
47 likely incomplete due to misassembly of the genome at a region with an exceptionally high  
48 G+C content of >80 mol%, which could not be resolved even by enormous sequencing  
49 efforts with standard methods. The updated capsid protein amino acid sequence now  
50 resembles those of RuV and ruhugu virus in size and harbours a predicted RNA binding  
51 domain that was not encoded in the original RusV genome version. The new sequence data  
52 indicate that RusV has the largest overall genome (9,631 nucleotides), intergenic region  
53 (290 nucleotides) and capsid protein-encoding sequence (331 codons) within the genus  
54 *Rubivirus*.

55 **(316 words; max. 350 words)**

## 56 Introduction

57 Rubella virus (RuV; species *Rubivirus rubellae*) was the sole member of the family  
58 *Matonaviridae* and the genus *Rubivirus* <sup>1</sup>, until recently its first relatives rustrela virus  
59 (RusV; *Rubivirus strelense*) and ruhugu virus (RuhV; *Rubivirus ruteetense*) were  
60 identified <sup>2</sup>. While RuhV was detected in apparently healthy cyclops leaf-nosed bats  
61 (*Hipposideros cyclops*) in Uganda, RusV was associated with cases of fatal neurological  
62 disease in placental and marsupial zoo animals in Germany. RusV was initially identified  
63 using a metagenomic sequencing workflow from brain tissues of a donkey (*Equus asinus*),  
64 a capybara (*Hydrochoeris hydrochaeris*), and a red-necked wallaby (*Macropus rufogriseus*)  
65 between July 2018 and October 2019 <sup>2,3</sup>. All of these animals were housed in a zoological  
66 garden located in northeast Germany, close to the Baltic Sea, and developed acute  
67 neurological signs such as ataxia and lethargy, which ultimately resulted in death. RusV  
68 was mainly detected in the central nervous system of these animals and only sporadically  
69 and in very low concentrations in extraneural organs. RusV-infected wild yellow-necked field  
70 mice (*Apodemus flavicollis*) were identified in close proximity to the encephalitic animals'  
71 housings. These rodents were considered as a likely reservoir host, as they carried the virus  
72 without obvious encephalitis whereas all tested individuals of other sympatrically occurring  
73 rodent species at the same location were RusV-negative <sup>2</sup>. However, the mode of  
74 transmission between potential reservoir and accidental spill-over hosts still remains to be  
75 identified <sup>2</sup>. Currently, no isolates of either RusV or RuhV are available and therefore, most  
76 data are limited to *in silico* predictions and analogies with RuV. Furthermore, sequencing  
77 of RusV from organ samples proved to be extremely difficult and only three full-length  
78 genome sequences and a few partial coding sequences are currently available (**Table 1**).

79 The genome of rubiviruses consists of single-stranded positive sense (+ss) RNA, that  
80 contains two open reading frames (ORF) encoding the non-structural p200 and structural  
81 p110 polyproteins, respectively <sup>4</sup>. Both ORFs are separated by an untranslated intergenic  
82 region (IGR). In RuV, co-translational cleavage of the p110 polyprotein results in three  
83 structural proteins E1, E2, and the capsid protein <sup>4</sup>. After cleavage by cellular signal  
84 peptidase, the capsid protein remains in the cytoplasm while E1 and E2 enter the secretory  
85 pathway using distinct translocation signals <sup>5</sup>. Based on sequence comparison with RuV,  
86 the genomes of RusV and RuhV are likewise predicted to encode the p110 polyprotein and  
87 the mature capsid, E1 and E2 proteins <sup>2</sup>. In RuV, the capsid protein consists of a structurally  
88 disordered N-terminal part that contains a RNA-binding domain (RBD) <sup>6</sup> and a structurally  
89 ordered C-terminal domain (CTD) <sup>7,8</sup> containing the E2 signal sequence <sup>9</sup>. While the

90 predicted capsid protein sequence and structure of RuhV is analogous to that of RuV, the  
91 capsid protein of RusV was considered enigmatic as it appears truncated and lacking e.g.  
92 the RBD <sup>10</sup>.

93 Here we analysed the RusV genome sequences from two novel cases of RusV encephalitis  
94 using a sophisticated target-specific capture enrichment with RNA baits prior to  
95 sequencing. This resulted in markedly improved virus-to-background sequence ratios and  
96 higher genome coverage particularly in regions of exceptionally high G+C ratios of  
97 >80 mol%. The *de novo* assembled sequences suggested a 309 nucleotide (nt) longer  
98 RusV genome sequence than initially reported. We also confirmed the sequence extension  
99 by reanalysing samples from previously published diseased animals and potential reservoir  
100 animals using the same methods, and finally solved the enigma of the unusual RusV capsid  
101 protein sequence. So far, the clinical and pathological data are limited to the first  
102 description of RusV fatal encephalitis <sup>2</sup>. We now present further clinical data and an in  
103 depth pathological and histopathological evaluation of the two new cases.

104

## 105 **Material and Methods**

### 106 **Animals and samples included in this study**

107 Brain samples were collected from a South American coati that was housed in a zoological  
108 garden in the Northern Germany, a wild Eurasian otter that was found nearby the zoo and  
109 three yellow-necked field mice that had been trapped during pest control measures at the  
110 zoo (**Table 1**). In addition, samples from previously published animals, including a donkey,  
111 a capybara and seven yellow-necked field mice, were re-analysed during this study <sup>2</sup>.

### 112 **Histopathology, immunohistochemistry and RusV RNA *in situ* hybridization**

113 Routine staining, immunohistochemistry (IHC) as well as RNA *in situ* hybridization (RNA ISH)  
114 was applied as described earlier with minimal adaptations summarized in Supplemental  
115 Table S1 (see also <sup>2</sup>). Briefly, formalin-fixed, paraffin-embedded (FFPE) brain tissues were  
116 processed for haematoxylin and eosin (HE) staining and examination using light  
117 microscopy. On consecutive slides, conventional Prussian Blue staining was used to  
118 demonstrate haemosiderin, whereas Luxol Fast Blue Cresyl Violet was applied for detection  
119 of myelin sheaths and Nissl substance. Immunohistochemistry was performed according  
120 to standardized procedures using markers to detect T-cells (CD3), B-cells (CD79a),  
121 microglial cells and macrophages (IBA1), astrocytes (glial fibrillary acidic protein, GFAP) and

122 apoptotic cells (active caspase 3). A bright red chromogen labelling was produced with 3-  
123 amino-9-ethylcarbazole substrate (AEC, DAKO). Sections were counterstained with Mayer's  
124 haematoxylin. RNA ISH was performed with the RNAScope 2-5 HD Reagent Kit-Red  
125 (Advanced Cell Diagnostics, USA) according to the manufacturer's instructions using a  
126 custom-designed probe against the RusV non-structural protein (p200, NSP) ORF, and a  
127 negative control probe against the dihydrodipicolinate reductase (*DapB*) gene. Analysis and  
128 interpretation were performed by a board-certified pathologist (AB).

### 129 **Total RNA extraction for sequencing**

130 Total RNA was extracted from frozen brain tissues as described previously <sup>11</sup>. Initially,  
131 approximately 20 - 30 mg of tissue was snap-frozen in liquid nitrogen and disintegrated  
132 using a cryoPREP impactor (Covaris, UK). The pulverized tissue was solubilized in pre-  
133 heated lysis buffer AL and RNA was extracted using the RNAdvance Tissue Kit (Beckman  
134 Coulter, Germany) in combination with a KingFisher Flex Purification System (Thermo  
135 Fisher Scientific, Germany).

### 136 **RusV-specific RT-qPCR**

137 RusV-specific RNA was detected by TaqMan RT-qPCR using the AgPath-ID One-Step RT-PCR  
138 reagents (Thermo Fisher Scientific, Germany) along with a modified primer/probe set  
139 targeting the p200 ORF <sup>2</sup>. Briefly, 2.5 µl extracted RNA was reverse-transcribed and  
140 amplified in a reaction mix of 12.5 µl total volume containing primers RusV\_1072\_A+  
141 (5'-CGAGCGYGTCTACAAGTTYA-3'; final concentration 0.8 µM) and RusV\_1237-  
142 (5'-GACCATGATGTTGGCGAGG-3'; 0.8 µM) and probe RusV\_1116\_A\_P  
143 (5'-[FAM]CCGAGGARGACGCCCTGTGC[BHQ1]-3'; 0.4 µM). The reaction was performed with  
144 the following cyclers setup: 45 °C for 10 min, 95 °C for 10 min, 45 cycles of 95 °C for 15  
145 sec, 60 °C for 30 sec and 72 °C for 30 sec on a Bio-Rad CFX96 qPCR cycler (Bio-Rad,  
146 Germany).

### 147 **Sequencing of total RNA**

148 Extracted total RNA was sequenced using a universal metagenomics sequencing  
149 workflow <sup>11, 12</sup>. An amount of 350 ng total RNA per sample was reverse-transcribed into  
150 cDNA using the SuperScript IV First-Strand cDNA Synthesis System (Invitrogen, Germany)  
151 and the NEBNext Ultra II Non-Directional RNA Second Strand Synthesis Module (New  
152 England Biolabs, Germany). Afterwards, cDNA was processed to generate Ion Torrent  
153 compatible barcoded sequencing libraries as detailed described <sup>2, 11</sup>. Libraries were

154 quantified with the QIAseq Library Quant Assay Kit (Qiagen, Germany) and subsequently  
155 sequenced on an Ion Torrent S5XL instrument using Ion 530 chips and chemistry for  
156 400 base pair reads (Thermo Fisher Scientific, Germany).

### 157 **Sequencing of rRNA-depleted and poly(A)+ enriched RNA**

158 For rRNA depletion we used the NEBNext rRNA Depletion Kit for human, mouse, and rat  
159 (New England Biolabs, USA) that specifically depletes cytoplasmic (5S, 5.8S, 18S and 28S  
160 rRNA) and mitochondrial ribosomal RNA (12S and 16S rRNA). As the depletion is rRNA  
161 sequence-specific, we first confirmed that the human-, mouse- and rat-specific panel would  
162 be compatible with samples from yellow-necked field mice by comparing available  
163 cytoplasmic and mitochondrial rRNA sequences of all species. Subsequently, 3 µg of the  
164 total RNA from two selected yellow-necked field mice were treated with the NEBNext rRNA  
165 Depletion Kit for human, mouse, and rat (New England Biolabs), following the  
166 manufactures instructions.

167 Enrichment of poly(A)+ RNA from total RNA was considered appropriate, as the RusV  
168 genome, like RuV<sup>13</sup>, comprises a poly(A) tail at the 3' terminus. For poly(A)+ enrichment,  
169 3 µg of total RNA from the same yellow-necked field mice were treated with the Dynabeads  
170 mRNA DIRECT Micro Purification Kit (Invitrogen, USA) following the manufacturer's  
171 instructions.

172 Both, rRNA depleted and poly(A)+ enriched RNA, were used for strand-specific library  
173 construction with the Colibri Stranded RNA Library Prep Kit (Thermo Fisher, USA). Libraries  
174 were quality-checked using a 4150 TapeStation System (Agilent Technologies, USA) with  
175 the High Sensitivity D1000 ScreenTape and reagents (Agilent Technologies) and were then  
176 quantified using a Qubit Fluorometer (Thermo Fisher) along with the dsDNA HS Assay Kit  
177 (Thermo Fisher). Libraries were pooled and sequenced on a NextSeq 500 (Illumina, USA)  
178 using a NextSeq 500/550 Mid-output Kit v2.5 with 300 cycles (Illumina).

### 179 **Design of custom panRubi bait panels**

180 All available whole-genome sequences of the genus *Rubivirus* were received from NCBI  
181 GenBank (86 RuV, one RuhV and three RusV sequences). The genome set was sent to  
182 Daicel Arbor Biosciences (Ann Arbor, USA) and a tailored custom myBaits panel for target  
183 enrichment via hybridization-based capture was designed. The resulting "panRubi" panel  
184 consists of 19,178 RNA oligonucleotide baits with a length of 60 nt arranged every 20 nt  
185 along the genomes (designated "panRubi bait set v1"). The set was later supplemented

186 with 22 additional baits covering the newly identified part of the capsid protein-encoding  
187 sequence and IGR arranged every 16 nt. This set was mixed with the “panRubi bait set v1”  
188 at a ratio of 1:10 to give the “panRubi bait set v2”. All bait sets were checked using Basic  
189 Local Alignment Search Tool (BLAST) search against human, mouse, horse, and opossum  
190 genomes and no BLAST hit was found.

### 191 **Application of RNA baits and sequencing**

192 The custom panRubi bait sets v1/v2 were applied to the sequencing libraries according to  
193 the manufacturer’s instructions (myBaits manual v.5.00, Arbor Biosciences, Sep 2020).  
194 Hybridization reactions were performed in 1.5 µl safe-lock tubes overlaid with one volume  
195 of mineral oil (Carl Roth, Germany), to keep the volume constant during hybridization using  
196 a ThermoMixer (Eppendorf, Germany) with 550 rotations per minute. We used the standard  
197 protocol (according to <sup>14</sup>) with a hybridization temperature of 65 °C and a hybridization time  
198 of about 24 hours. The enriched and purified samples were amplified using the GeneRead  
199 DNA Library L amplification Kit (Qiagen, Germany) according to manufacturer’s instructions  
200 with 14 cycles and amplicons were purified using solid-phase paramagnetic bead  
201 technology. Treated libraries were sequenced after quality check using a Bioanalyzer 2100  
202 (Agilent Technologies) and quantification as described above.

### 203 **Read processing and *de novo* assembly**

204 Ion Torrent-derived reads from the myBaits capture enrichment approach were initially  
205 quality-trimmed and specific adapters were removed using the 454 Sequencing Systems  
206 Software (version 3.0). Instead of host/background removal using specific reference  
207 sequences, a G+C content filter was applied to the trimmed reads, as the RusV genome  
208 has a particularly high average G+C content of 70.6 mol% <sup>2</sup>. In detail, only reads with an  
209 average G+C content of ≥60 mol% were filtered using PRINSEQ-lite (version 0.20.4; <sup>15</sup>) and  
210 subsequently used for *de novo* assembly with SPAdes genome assembler (version  
211 3.15.2; <sup>16</sup>) running in single cell mode (–sc) for Ion Torrent data (–iontorrent). The resulting  
212 contigs were mapped to the RusV reference sequence MN552442 using Geneious generic  
213 mapper (Geneious Prime 2021.0.1) with medium sensitivity allowing discovery of structural  
214 variants, and short insertions/deletions (indels) of any size. A consensus sequence was  
215 generated and reads were finally mapped back to the consensus sequence using Geneious  
216 generic mapper in order to manually inspect genomic termini and possible frameshifts  
217 caused by homopolymers.

218 Illumina-derived reads from rRNA-depleted and poly(A)+-enriched RNA were initially  
219 trimmed using Trim Galore (version 0.6.6; <sup>17</sup>) with automated adapter selection and reads  
220 containing only poly(A) homopolymers were trimmed using BBDuk/BBMap (version 38.18, <sup>18</sup>). For coverage analysis, the trimmed reads of each sample were mapped  
221 to the respective assembled genome using Geneious Prime generic mapper in “Low  
222 Sensitivity / Fastest” mode. The indexed BAM files were then processed with SAMtools  
223 depth (version 1.11; <sup>19</sup>).

## 225 **Phylogenetic analysis**

226 Complete RusV genome sequences were aligned using MAFFT (version 7.450; <sup>20</sup>) and then  
227 used as input for approximately-maximum-likelihood reconstruction with Fast Tree  
228 (version 2.1.11; <sup>21</sup>) using the generalized time-reversible (GTR) model with 5 rate  
229 categories and optimized Gamma20 likelihood. The resulting tree was inspected using  
230 Geneious Prime (version 2021.0.1).

231

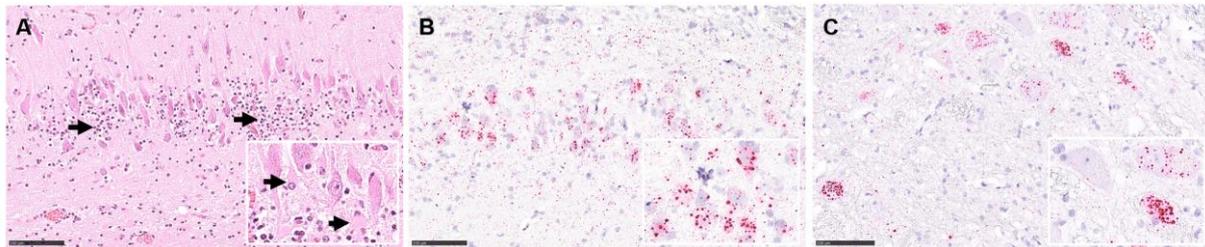
## 232 **Results**

### 233 **Two carnivoran mammals with neurological disorder**

234 In August 2020, a South American coati (*Nasua nasua*) kept in the zoo showed lethargy,  
235 hind limb weakness, convulsion and tremor. Two days later and finally unmoving, the  
236 animal was euthanized. Gross pathology revealed swelling of the liver and hyperkeratosis  
237 of the footpads. Initial histopathology identified a non-suppurative meningoencephalitis.  
238 Findings in the liver included scattered single cell necrosis of hepatocytes and minimal  
239 microvesicular fatty change interpreted to be clinically irrelevant, while the footpad  
240 hyperkeratosis was interpreted to be age-related. Standard diagnostic tests were negative  
241 for mammalian bornaviruses, canine distemper virus and *Salmonella* spp.

242 In December 2020, a wild Eurasian otter (*Lutra lutra*) was found in the vicinity of the very  
243 same zoological garden, without any reported link to the zoo areal, showing abnormal  
244 movement. Prior to capturing, the animal was observed in the open waters of the nearby  
245 Baltic Sea coast and then later found on the premises of a local school. The animal was  
246 sent for clinical observation to the zoo, presenting in a state of malnourishment but with  
247 increased food and water uptake, loss of natural shyness and an abrasion at the head  
248 indicating blunt trauma. Abnormal movements were still present until the animal was found  
249 dead three days later. Pathological examination confirmed hairless spots at the head with

250 a focal perforation of the skin but otherwise non-specific alterations interpreted to be  
251 associated with acute, agonal cardiovascular failure. Initial routine histology identified a  
252 non-suppurative meningoencephalitis but no further lesions in other organs. Standard  
253 diagnostic tests were negative for mammalian bornaviruses, influenza A virus, canine  
254 distemper virus, rabies virus, *Salmonella* spp. and *Toxoplasma gondii*.



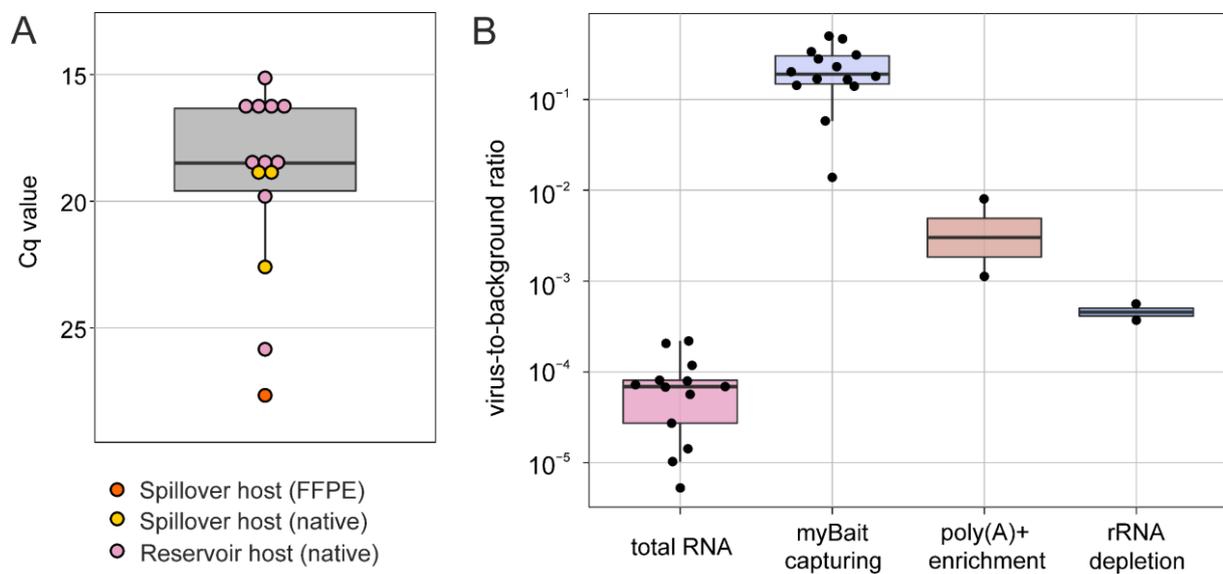
255  
256 **Fig. 1:** Histopathology from cases of rustrela virus (RusV)-associated meningoencephalitis in a  
257 Eurasian otter (*Lutra lutra*) and a South American coati (*Nasua nasua*). (A) Non-suppurative  
258 meningoencephalitis in the hippocampus region of the otter, with mononuclear infiltrates  
259 (arrows) and loss of Nissl substance indicating neuronal degeneration (inlay with arrows), HE  
260 stain. Detection of RusV RNA in neurons of the hippocampus region of the otter (B and inlay) and  
261 brain stem of the coati (C and inlay). RNA ISH, chromogenic labelling (fast red) with probes to  
262 RusV non-structural polyprotein encoding region, Mayer's hematoxylin counter stain. Scale bar  
263 100  $\mu$ m.

#### 264 Histopathology confirms RusV-associated encephalitis

265 In general, follow-up histopathology of the RusV-infected South American coati and  
266 Eurasian otter confirmed our results previously reported for the RusV-infected donkey,  
267 capybara and wallaby from the same zoo. Associated with a non-suppurative  
268 meningoencephalitis (Fig. 1A), RNA ISH confirmed the presence of RusV-specific RNA  
269 within neuronal cell bodies and their processes in both animals (Fig. 1B and C). Routine HE  
270 staining (Fig. 1A) as well as Luxol fast blue Cresyl violet staining (Supplemental Fig. S1A)  
271 identified neuronal degeneration in the brain of the Eurasian otter but not the South  
272 American coati. Scattered cells, in particular perivascularly, were active caspase 3-labelled,  
273 indicating subtle apoptosis induction (Supplemental Fig. S1B). Multifocal perivascular cells  
274 in brain samples from the otter were positive for iron in the Prussian Blue reaction,  
275 confirming intravital hemorrhages (Supplemental Fig. S1C), potentially associated with a  
276 suspected history of a blunt trauma. The non-suppurative meningoencephalitis was  
277 characterized by perivascular and disseminated infiltrates and few microglial nodules.  
278 Immunohistochemistry identified mainly infiltrating CD3-positive T-cells (Supplemental  
279 Fig. S1D) but only single CD79-labelled B-cells (Supplemental Fig. S1D inlay). Numerous  
280 IBA1-positive microglial cells and infiltrating macrophages were detected intralasionally  
281 (Supplemental Fig. S1E). In addition, GFAP immunohistochemistry indicated activation of  
282 astrocytes, exhibiting a plump cell shape (Supplemental Fig. S1F).

## 283 RT-qPCR confirms presence of RusV in encephalitic animals and reservoir hosts

284 A RusV-specific RT-qPCR confirmed the presence of viral RNA in the brain of the South  
285 American coati (Cq 18.9) and the Eurasian otter (Cq 22.5). Furthermore, using the same  
286 RT-qPCR setup, we also reanalysed samples from two previously investigated zoo animals  
287 and from ten previously published or recently collected RusV-infected wild yellow-necked  
288 field mice from within and around the zoo (**Table 1**). Cq values of frozen brain samples  
289 ranged from 15.1 to 25.8, with a median of 18.4 (**Fig. 2A**), whereas FFPE brain tissue from  
290 a capybara with encephalitis revealed the highest Cq of 27.6 corresponding to the lowest  
291 amount of detectable RNA.



292

293 **Fig. 2: (A)** Comparison of RusV-specific RT-qPCR Cq values for frozen or FFPE brain samples from  
294 potential reservoir (yellow-necked field mouse) and spill-over hosts (donkey, capybara, South  
295 American coati, European otter). **(B)** Comparison of virus-to-background sequence ratio observed  
296 in sequencing data using different RNA preparations and post library capturing methods.

## 297 Increasing RusV sequencing efficiency

298 Initially, we used total RNA from brain samples of the Eurasian otter and South American  
299 coati for sequencing and *de novo* assembly. However, this resulted in incomplete and  
300 highly fragmented genome sequences due to very low virus-to-background sequence ratios  
301 of 0.021% and 0.001% for the South American coati and Eurasian otter, respectively. The  
302 virus-to-background ratios observed during sequencing of total RNA from all samples  
303 included in this study (**Table 1**) ranged from 0.00053% to 0.022%, with a median of  
304 0.0069% (**Fig. 2B**). To increase the efficiency of RusV sequencing, we compared poly(A)+  
305 enrichment, rRNA depletion as well as post library hybridization-based capturing (bait  
306 capturing) for selected samples.

307 By reducing host-derived RNA, poly(A)+ enrichment and rRNA depletion increased virus-to-  
308 background sequence ratios by factors of 67 and 6.8, respectively, as compared to total  
309 RNA, resulting in median virus-to-background sequence ratios of 0.46% and 0.047%,  
310 respectively (**Fig. 2B**). The application of bait capturing to libraries prepared from total RNA  
311 achieved virus-to-background sequence ratios of 1.4% to 49.9% with a median of 19.1%  
312 (**Fig. 2B**), corresponding to a median 2,772-fold increase.

313 The characteristic sequence coverage pattern observed for bait-captured libraries closely  
314 resembled that of total RNA sequencing (Supplemental Fig. S2). In contrast, libraries from  
315 poly(A)+ enriched RNA had a strong bias in coverage towards the 3' end of the RusV  
316 genome. Depletion of rRNA resulted in a relatively uniform coverage across the genome  
317 with a bias towards the 5' end of the genome (Supplemental Fig. S2). No coverage dropout  
318 was noted for any of the applied methods.

### 319 **Generation and comparison of full length RusV genomes**

320 As bait capturing proved to be very efficient, we applied the technique to all 14 available  
321 brain samples (**Table 1**), including South American coati and Eurasian otter, and used the  
322 sequencing data for *de novo* assembly. The assembly of each library resulted in contigs  
323 that were matched to the RusV genome MN552442. For all samples, a full-length RusV  
324 genome without any gaps could be derived from the matched *de novo* assembled contigs.

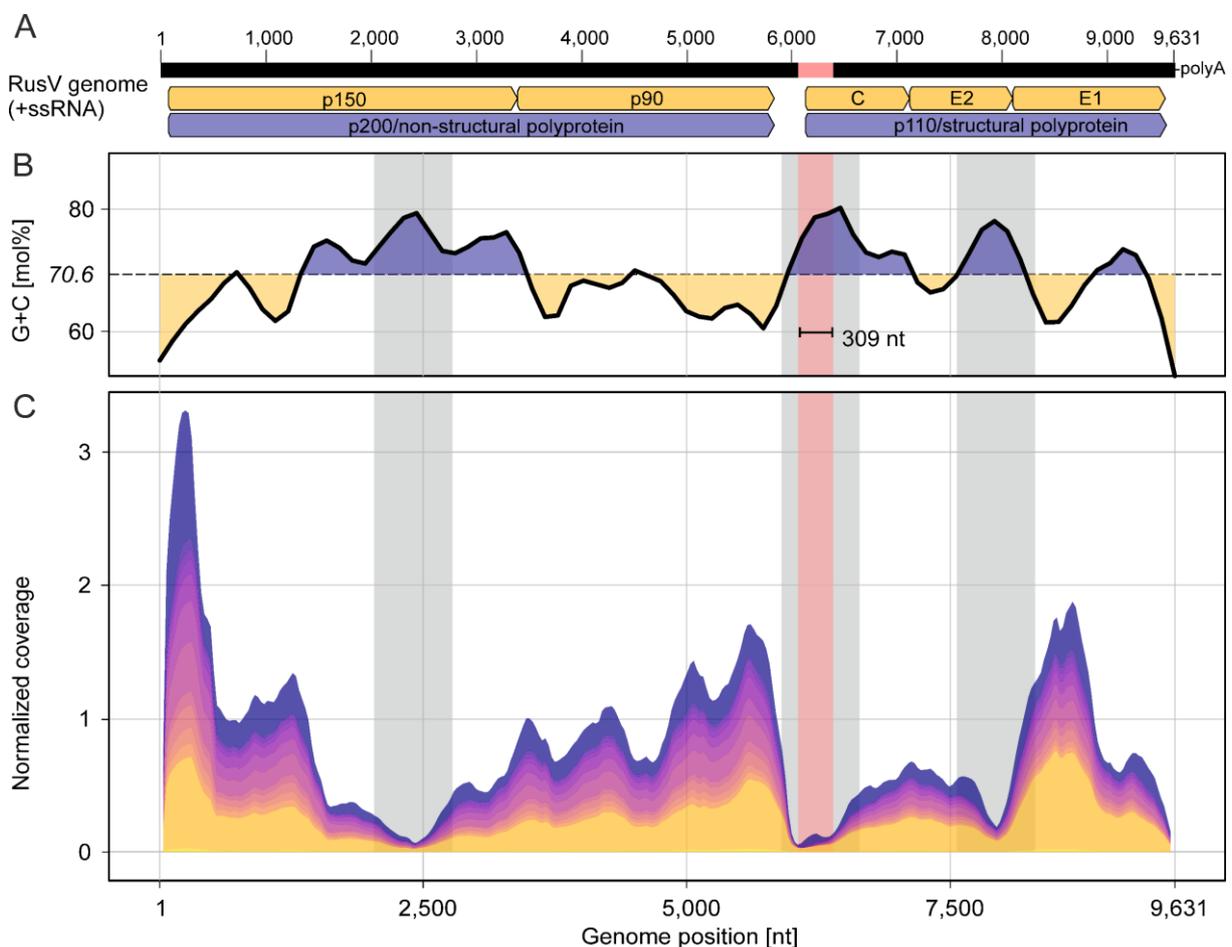
325 An alignment of all 14 RusV genome sequences showed only minor variation with an overall  
326 pairwise nt identity of 98.8%. Phylogeny based on the aligned whole genome sequences  
327 confirmed the high genetic identity of the RusV genomes originating from within or in close  
328 proximity of the zoo (Supplemental Fig. S3). RusV sequences from apparently healthy  
329 yellow-necked field mice and encephalitic mammals, including the South American coati  
330 and Eurasian otter, clustered closely together. Notably, two RusV sequences from yellow-  
331 necked field mice collected in a distance of about 10 km from the zoo grouped in a  
332 separate genetic branch (Supplemental Fig. S3).

### 333 **Revised RusV genome and implications for the IGR and capsid protein-coding sequence**

334 All 14 RusV genomes assembled from bait-captured libraries showed a 309 nt stretch  
335 ranging from pos. 6,062 to pos. 6,370 and covering part of the IGR and the N-terminal part  
336 of the capsid protein-coding sequence. This stretch had not been present in the three  
337 initially released RusV genomes generated by total RNA sequencing<sup>2</sup> but was now  
338 identified when re-sequencing the very same sample materials using bait capturing

339 (Fig. 3A). As the panRubi v1 bait set did not comprise the extra 309 nt-long region, the bait  
340 set was complemented with probes specifically targeting this region, leading to a further  
341 improved coverage within the respective region (Supplemental Fig. S4).

342 In general, the observed sequencing coverage varied considerably across the genome,  
343 showing pronounced maxima and minima in all samples (Fig. 3C). The three genomic  
344 regions with the most prominent reduction in sequence coverage correlated with the  
345 highest G+C content (Fig. 3B and C), while genome regions with very high coverage  
346 correlated with lower G+C content. The newly identified 309 nt region correlated with a  
347 G+C peak and possessed a particularly low sequence coverage (Fig. 3A-C).



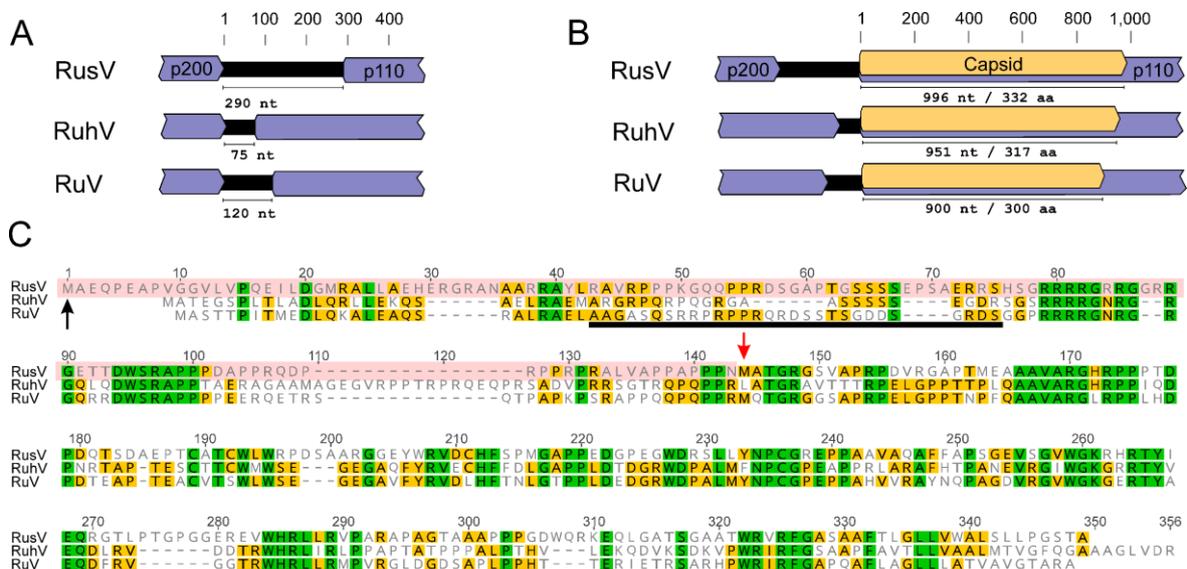
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349 **Fig. 3:** Schematic rustrela virus (RusV) genome sequence (A) showing averaged G+C content (B)  
350 and cumulated RusV sequence coverage of all 14 animals included in this study (C). The newly  
351 identified 309 nt sequence stretch partly covering the intergenic region and p110 ORF is  
352 highlighted in red. Note that the start of the p110 coding ORF is located within the newly identified  
353 sequence stretch, leading to a longer capsid protein sequence as compared to the previously  
354 published RusV genomes. Grey labelled areas in B and C indicate areas of particularly high G+C  
355 content.

356

357 As a consequence, the IGR and the predicted p110 ORF are longer than initially reported  
 358 (Fig. 4A and B). The IGR of all 14 full-length RusV sequences is spanning 290 nt between  
 359 the stop codon of the predicted p200 ORF and start codon of the p110 ORF. In comparison,  
 360 the IGRs of RuhV and RuV were reported to be 75 nt and 120 nt in length, respectively  
 361 (Fig. 4A). Based on an ATG start codon in the newly identified 309 nt region and a prediction  
 362 of the signal peptidase cleavage site (Supplemental Fig. S5), the predicted capsid protein-  
 363 encoding sequence of RusV is 996 nt (332 amino acids, aa) in length. In comparison, the  
 364 length of the capsid protein-encoding regions of RuhV and RuV was predicted to be 951 nt  
 365 (317 aa) and 900 nt (300 aa), respectively (Fig. 4B).

366 Comparison of the revised capsid protein sequence of RusV to both RuhV and RuV revealed  
 367 highly conserved stretches (Fig. 4C). The revised RusV capsid protein sequence comprised  
 368 a part that has been predicted to be the RBD in RuV. This part had been absent in the  
 369 initially published RusV genome. Directly downstream of the predicted RuV RBD region, a  
 370 polybasic motif (RRRRG R/N RG) can be found that is highly conserved between RusV,  
 371 RuhV and RuV. This polybasic motif is followed by a likewise highly conserved hydrophobic  
 372 motif (DWSRAPP).



373

374 **Fig. 4:** Comparison of the rubivirus intergenic region and capsid protein-encoding sequences. (A)  
 375 The size of the intergenic region between the non-structural (p200) and structural polyprotein  
 376 (p110) ORFs of rustrela virus (RusV; MN552442.2), ruhugu virus (RuhV; MN547623) and rubella  
 377 virus (RuV; NC\_001545) is shown. (B) The predicted length of the capsid protein-coding sequence  
 378 (highlighted in orange) is shown for RusV, RuhV and RuV. (C) The sequences of the capsid protein  
 379 from RusV, RuhV and RuV are compared using an amino acid sequence alignment. Amino acid  
 380 residues highlighted in green or yellow are conserved in all three or at least in two of the viruses,  
 381 respectively. The N-terminal part of the RusV mature capsid protein (highlighted in red; start  
 382 marked by black arrow) has been determined in this study. The red arrow indicates the predicted  
 383 start of the capsid protein in the previously published RusV sequence. The RNA binding site of the  
 384 RuV capsid protein is indicated by the black bar.

## 385 Discussion

386 We recently discovered RusV in the central nervous system of encephalitic zoo animals and  
387 wild yellow-necked field mice on the basis of metagenomic sequencing of total RNA, RT-  
388 qPCR and RNA ISH <sup>2</sup>. More than 300 million reads from different sequencing platforms and  
389 numerous samples and subsamples were used in order to generate the first RusV genomes  
390 originating from three individuals (MN552442.1, MT274724.1 and MT274725.1). A  
391 combination of *de novo* assembly, mapping, BLASTx, and manual inspection was used to  
392 generate these RusV genomes <sup>2</sup>. The assembly was exceptionally difficult, as most parts of  
393 the RusV genome have a G+C content of >70 mol% with low complexity G+C stretches and  
394 coverage dropping drastically at several positions (**Fig. 3B** and **C**). In the IGR, the G+C  
395 content even exceeds 85 mol%. Nevertheless, despite very large efforts in sequence  
396 determination and characterisation of the RusV genome, questions remained regarding its  
397 unusual IGR and capsid protein, which appeared to be rather short in comparison to RuV  
398 and RusV and lacked a potential RBD <sup>10</sup>.

399 We now investigated two new cases of RusV-associated fatal meningoencephalitis in a  
400 European otter and a South American coati that clinically and histologically closely  
401 resembled previous RusV-associated cases and that further broadened the spectrum of  
402 infected mammals, which now includes placental mammals of the orders Rodentia  
403 (families Caviidae and Muridae), Carnivora (families Procynoidae and Mustelidae), and  
404 Perissodactyla (family Equidae) as well as marsupials of the order Diprotodontia (family  
405 Macropodidae). This broad host spectrum is in clear contrast to rubella virus, for which  
406 humans are the only host <sup>8</sup>. So far, we assume that the wild yellow-necked field mouse may  
407 act as reservoir host. However, the transmission route to the other hosts remains unclear.

408 Despite the relatively low RusV-specific RT-qPCR Cq values in native organ samples,  
409 detected using a RusV-specific RT-qPCR (**Fig. 2A**), the virus-to-host sequence ratio observed  
410 when sequencing total RNA was unsatisfying. It has been shown, that genome length, virus  
411 species, virus- and host-derived RNA concentrations as well as the overall composition of  
412 the sample matrix impact the virus-background ratio <sup>22</sup>. RT-qPCR results often do not reflect  
413 this complex interplay and may lead to false expectations for sequencing.

414 As sequencing of RusV genomes from total RNA proved to be very difficult, we attempted  
415 to increase the sequencing efficiency by poly(A)+ enrichment, rRNA depletion and post-  
416 library bait capturing. Poly(A)+ enrichment was more efficient than rRNA depletion,  
417 resulting in higher virus-to-background ratios (**Fig. 2B**). This observation is in accordance

418 with other studies, in which rRNA-depleted RNA preparations contained many host-derived  
419 small or long non-coding RNAs that are absent in poly(A)+ enriched RNA preparations <sup>23</sup>.  
420 However, poly(A)+ selection introduced a 3' sequence coverage bias resulting in poor  
421 5' coverage. This bias has been reported previously for poly(A)+ enrichment methods and  
422 is most likely caused by partially degraded transcripts particularly in samples with highly  
423 degraded RNA <sup>24</sup>. RNA quality and integrity plays a major role in sequencing experiments  
424 and is directly connected to sampling conditions, transportation and storage <sup>25-27</sup>. While  
425 we used qualified and robust methods for RNA extraction and preservation <sup>11</sup>, the RNA  
426 preparations used for comparison of the different methods originated from brain tissues  
427 of two wild-trapped rodents that were sampled under suboptimal conditions.

428 Hybridization-based capturing has been shown to markedly increase efficiency of RNA virus  
429 sequencing previously <sup>14, 28-30</sup> and was also found to be most efficient in this study,  
430 increasing the median virus-to-background sequence ratio 2,772-fold. Using this  
431 technique, we sequenced or re-sequenced 14 full-length RusV genomes from cases of  
432 encephalitis and from wild yellow-necked field mice. Using the hybridization-based bait  
433 capturing method, the overall sequence coverage and especially the coverage in  
434 challenging regions was markedly improved. However, we found a correlation between  
435 sharp drops in sequencing coverage within regions of very high G+C content exceeding  
436 ~75 mol%. This may indicate a technical limit of the used sequencing platforms and has  
437 been described for different technologies <sup>31-34</sup>. It has also been suggested, that extreme  
438 G+C contents may negatively affect *de novo* assemblies <sup>35</sup>.

439 Within a region of high G+C content, spanning IGR and the 5'-end of the capsid protein-  
440 encoding sequence, we now found a notable sequence difference, namely a previously  
441 unidentified stretch of 309 nt, in comparison to the initially reported RusV genome.  
442 Thereby, the predicted capsid protein of RusV is longer than described earlier and now  
443 includes the typical rubivirus capsid protein features, such as the RBD that might be crucial  
444 for virion formation <sup>6, 7</sup>. The RBD was unexpectedly missing in the initially annotated RusV  
445 capsid protein as pointed out recently in detail by Das and Kilian <sup>10</sup>. An alignment of the  
446 capsid proteins of RusV, RuhV and RuV showed highly conserved motifs that were initially  
447 absent in the predicted RusV protein. However, the region identified as RBD in RuV <sup>6</sup>  
448 appears to be only poorly conserved on aa sequence level in the RuhV or RusV when  
449 compared in an alignment. Whether conserved motifs are involved in RNA binding or other  
450 structural features remains unclear, as no structural model is currently available for the N-  
451 terminal part of the RuV capsid protein <sup>7</sup>.

452 The revised version of the RusV genome reveals RusV to have the longest capsid protein-  
453 encoding sequence and IGR among all three currently known matonaviruses (**Fig. 2A** and  
454 **B**). It has been shown for RuV, that the p110 polyprotein is translated from a subgenomic  
455 RNA by using a separate promoter within the IGR<sup>36, 37</sup>. However, based on the coverage  
456 along the genome, we could not find any indication for the presence of subgenomic RNA in  
457 the analysed samples. This may indicate that either RusV does not translate the p110  
458 polyprotein from a subgenomic RNA or the RusV replication cycle includes stages without  
459 presence of subgenomic RNA. Future studies should address these open questions.

460 In conclusion, we were able to markedly increase RusV sequencing efficiency leading to an  
461 improved genome coverage by employing a bait capturing-based enrichment strategy.  
462 Overall, 14 high-quality whole-genomes from RusV-related encephalitis cases and reservoir  
463 hosts could be generated applying this strategy. By *de novo* assembly, we identified an  
464 extra 309 nt sequence spanning the partial RusV IGR and 5'-end of the capsid protein-  
465 encoding region. The RusV example impressively demonstrates the difficulties in correctly  
466 determining sequences with an extreme G+C content, but also suggests possible solutions  
467 that are now available, such as targeted enrichment via RNA baits. The updated RusV  
468 sequence now allows further studies about the function of conserved regions of RusV, but  
469 also about viral replication using reverse genetics.

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## 474 **Data availability**

475 Revised versions of previously published RusV genome sequences are available under  
476 DDBJ/ENA/GenBank accession numbers MN552442.2, MT274724.2, and MT274725.2.  
477 Novel RusV genomes from this study are available under DDBJ/ENA/GenBank accession  
478 numbers: OL960716 - OL960726

## 479 **Ethics statement**

480 This study involved no animal experiments. All animal materials were from routine  
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## 486 Competing interests

487 The authors declare no competing interests.

## 488 Author contributions

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491 **Formal analysis:** FP, AB

492 **Investigation:** FP, AB, DR, SN, CB, SG, CL, CW, AE, DH

493 **Supervision:** DR, MB, RGU

494 **Visualization:** FP, AB

495 **Writing - Original Draft:** FP, AB, DR

496 **Writing - Review & Editing:** FP, AB, DR, SN, CB, SG, CL, CW, AE, DH, RGU, MB

497

## 498 References

- 499 1. Rubing Chen, Suchetana Mukhopadhyay, Andres Merits, Bethany Bolling, Farooq  
500 Nasar, Lark L. Coffey, Ann Powers, Scott C. Weaver, Donald Smith, Peter Simmonds  
501 and Stuart Siddell (2018) Create a new family *Matonaviridae* to include the genus  
502 *Rubivirus*, removed from the family *Togaviridae*.  
503 <https://talk.ictvonline.org/ictv/proposals/2018.013S.A.v3.Matonaviridae.zip>.  
504 Accessed 28 Jul 2021
- 505 2. Bennett AJ, Paskey AC, Ebinger A et al. (2020) Relatives of rubella virus in diverse  
506 mammals. *Nature* 586:424–428. <https://doi.org/10.1038/s41586-020-2812-9>
- 507 3. Bennett AJ, Paskey AC, Ebinger A et al. (2020) Author Correction: Relatives of rubella  
508 virus in diverse mammals. *Nature* 588:E2. <https://doi.org/10.1038/s41586-020-2897-1>  
509
- 510 4. Oker-Blom C (1984) The gene order for rubella virus structural proteins is NH2-C-E2-  
511 E1-COOH. *J Virol* 51:354–358

- 512 5. Oker-Blom C, Jarvis DL, Summers MD (1990) Translocation and cleavage of rubella  
513 virus envelope glycoproteins: identification and role of the E2 signal sequence. *J Gen*  
514 *Virol* 71 (Pt 12):3047–3053. <https://doi.org/10.1099/0022-1317-71-12-3047>
- 515 6. Liu Z, Yang D, Qiu Z et al. (1996) Identification of domains in rubella virus genomic  
516 RNA and capsid protein necessary for specific interaction. *J Virol* 70:2184–2190
- 517 7. Mangala Prasad V, Willows SD, Fokine A et al. (2013) Rubella virus capsid protein  
518 structure and its role in virus assembly and infection. *Proc Natl Acad Sci U S A*  
519 110:20105–20110. <https://doi.org/10.1073/pnas.1316681110>
- 520 8. Das PK, Kielian M (2021) Molecular and Structural Insights into the Life Cycle of  
521 Rubella Virus. *J Virol*. <https://doi.org/10.1128/JVI.02349-20>
- 522 9. Suomalainen M, Garoff H, Baron MD (1990) The E2 signal sequence of rubella virus  
523 remains part of the capsid protein and confers membrane association in vitro. *J Virol*  
524 64:5500–5509. <https://doi.org/10.1128/JVI.64.11.5500-5509.1990>
- 525 10. Das PK, Kielian M (2021) The Enigmatic Capsid Protein of an Encephalitic Rubivirus.  
526 *J Virol*. <https://doi.org/10.1128/JVI.02294-20>
- 527 11. Wylezich C, Papa A, Beer M et al. (2018) A Versatile Sample Processing Workflow for  
528 Metagenomic Pathogen Detection. *Sci Rep* 8. [https://doi.org/10.1038/s41598-018-](https://doi.org/10.1038/s41598-018-31496-1)  
529 [31496-1](https://doi.org/10.1038/s41598-018-31496-1)
- 530 12. Forth LF, Höper D (2019) Highly efficient library preparation for Ion Torrent sequencing  
531 using Y-adapters. *Biotechniques* 67:229–237. [https://doi.org/10.2144/btn-2019-](https://doi.org/10.2144/btn-2019-0035)  
532 [0035](https://doi.org/10.2144/btn-2019-0035)
- 533 13. Dominguez G, Wang C-Y, Frey TK (2004) Sequence of the genome RNA of rubella virus:  
534 Evidence for genetic rearrangement during togavirus evolution. *Virology* 177:225–  
535 238. [https://doi.org/10.1016/0042-6822\(90\)90476-8](https://doi.org/10.1016/0042-6822(90)90476-8)
- 536 14. Wylezich C, Calvelage S, Schlottau K et al. (2021) Next-generation diagnostics: virus  
537 capture facilitates a sensitive viral diagnosis for epizootic and zoonotic pathogens  
538 including SARS-CoV-2. *Microbiome* 9:51. [https://doi.org/10.1186/s40168-020-](https://doi.org/10.1186/s40168-020-00973-z)  
539 [00973-z](https://doi.org/10.1186/s40168-020-00973-z)
- 540 15. Schmieder R, Edwards R (2011) Quality control and preprocessing of metagenomic  
541 datasets. *Bioinformatics* 27:863–864.  
542 <https://doi.org/10.1093/bioinformatics/btr026>
- 543 16. Bankevich A, Nurk S, Antipov D et al. (2012) SPAdes: a new genome assembly  
544 algorithm and its applications to single-cell sequencing. *J Comput Biol* 19:455–477.  
545 <https://doi.org/10.1089/cmb.2012.0021>

- 546 17. Felix Krueger, Frankie James, Phil Ewels et al. (2021) TrimGalore: v0.6.7.  
547 <https://github.com/FelixKrueger/TrimGalore>. Accessed 28 Jul 2021
- 548 18. Brian Bushnell BBTools suite. <https://jgi.doe.gov/data-and-tools/bbtools/>. Accessed  
549 23 Jul 2021
- 550 19. Li H, Handsaker B, Wysoker A et al. (2009) The Sequence Alignment/Map format and  
551 SAMtools. *Bioinformatics* 25:2078–2079.  
552 <https://doi.org/10.1093/bioinformatics/btp352>
- 553 20. Katoh K, Standley DM (2013) MAFFT Multiple Sequence Alignment Software Version  
554 7: Improvements in Performance and Usability. *Mol Biol Evol* 30:772–780.  
555 <https://doi.org/10.1093/molbev/mst010>
- 556 21. Price MN, Dehal PS, Arkin AP (2010) FastTree 2 – approximately maximum-likelihood  
557 trees for large alignments. *PLoS One* 5:e9490.  
558 <https://doi.org/10.1371/journal.pone.0009490>
- 559 22. Ebinger A, Fischer S, Höper D (2021) A theoretical and generalized approach for the  
560 assessment of the sample-specific limit of detection for clinical metagenomics.  
561 *Comput Struct Biotechnol J* 19:732–742.  
562 <https://doi.org/10.1016/j.csbj.2020.12.040>
- 563 23. Zhao S, Zhang Y, Gamini R et al. (2018) Evaluation of two main RNA-seq approaches  
564 for gene quantification in clinical RNA sequencing: polyA+ selection versus rRNA  
565 depletion. *Sci Rep* 8:4781. <https://doi.org/10.1038/s41598-018-23226-4>
- 566 24. Cui P, Lin Q, Ding F et al. (2010) A comparison between ribo-minus RNA-sequencing  
567 and polyA-selected RNA-sequencing. *Genomics* 96:259–265.  
568 <https://doi.org/10.1016/j.ygeno.2010.07.010>
- 569 25. Sidova M, Tomankova S, Abaffy P et al. (2015) Effects of post-mortem and physical  
570 degradation on RNA integrity and quality. *Biomol Detect Quantif* 5:3–9.  
571 <https://doi.org/10.1016/j.bdq.2015.08.002>
- 572 26. Bauer M, Gramlich I, Polzin S et al. (2003) Quantification of mRNA degradation as  
573 possible indicator of postmortem interval – a pilot study. *Leg Med (Tokyo)* 5:220–227.  
574 <https://doi.org/10.1016/j.legalmed.2003.08.001>
- 575 27. Bonadio RS, Nunes LB, Moretti PNS et al. (2021) Insights into how environment  
576 shapes post-mortem RNA transcription in mouse brain. *Sci Rep* 11:13008.  
577 <https://doi.org/10.1038/s41598-021-92268-y>
- 578 28. Metsky HC, Matranga CB, Wohl S et al. (2017) Zika virus evolution and spread in the  
579 Americas. *Nature* 546:411–415. <https://doi.org/10.1038/nature22402>

- 580 29. Piantadosi A, Kanjilal S, Ganesh V et al. (2018) Rapid Detection of Powassan Virus in  
581 a Patient With Encephalitis by Metagenomic Sequencing. *Clin Infect Dis* 66:789–792.  
582 <https://doi.org/10.1093/cid/cix792>
- 583 30. Matranga CB, Andersen KG, Winnicki S et al. (2014) Enhanced methods for unbiased  
584 deep sequencing of Lassa and Ebola RNA viruses from clinical and biological samples.  
585 *Genome Biol* 15:519. <https://doi.org/10.1186/PREACCEPT-1698056557139770>
- 586 31. Browne PD, Nielsen TK, Kot W et al. (2020) GC bias affects genomic and metagenomic  
587 reconstructions, underrepresenting GC-poor organisms. *Gigascience* 9.  
588 <https://doi.org/10.1093/gigascience/giaa008>
- 589 32. Dohm JC, Lottaz C, Borodina T et al. (2008) Substantial biases in ultra-short read data  
590 sets from high-throughput DNA sequencing. *Nucleic Acids Res* 36:e105.  
591 <https://doi.org/10.1093/nar/gkn425>
- 592 33. Quail MA, Smith M, Coupland P et al. (2012) A tale of three next generation sequencing  
593 platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq  
594 sequencers. *BMC Genomics* 13:341. <https://doi.org/10.1186/1471-2164-13-341>
- 595 34. Benjamini Y, Speed TP (2012) Summarizing and correcting the GC content bias in high-  
596 throughput sequencing. *Nucleic Acids Res* 40:e72.  
597 <https://doi.org/10.1093/nar/gks001>
- 598 35. Chen Y-C, Liu T, Yu C-H et al. (2013) Effects of GC bias in next-generation-sequencing  
599 data on de novo genome assembly. *PLoS One* 8:e62856.  
600 <https://doi.org/10.1371/journal.pone.0062856>
- 601 36. Tzeng W-P, Frey TK (2002) Mapping the rubella virus subgenomic promoter. *J Virol*  
602 76:3189–3201. <https://doi.org/10.1128/JVI.76.7.3189-3201.2002>
- 603 37. Oker-Blom C, Ulmanen I, Kääriäinen L et al. (1984) Rubella virus 40S genome RNA  
604 specifies a 24S subgenomic mRNA that codes for a precursor to structural proteins. *J*  
605 *Virol* 49:403–408

606 **Table 1:** Rustrela virus-infected zoo animals, Eurasian otter and yellow-necked field mice from Northern Germany included in this study

| Strain   | Organism                         | Sampling date | Location               | Study                     | Sequence data# | Previous accessions    | Updated and new full genome accession |
|--|----------------------------------|---------------|------------------------|---------------------------|----------------|------------------------|---------------------------------------|
| Yellow-necked field mouse/Mu09-1341/2009/Germany | <i>Apodemus flavicollis</i>      | Jul 2009      | ~2 km distance to zoo  | Bennet <i>et al.</i> 2020 | T, B           | MT274737.1, MT274731.1 | OL960721                              |
| Donkey/19_041-1/2019/Germany                     | <i>Equus asinus</i>              | Mar 2019      | housed in zoo          | Bennet <i>et al.</i> 2020 | T, B           | MN552442.1             | MN552442.2                            |
| Capybara/P19-643/2019/Germany                    | <i>Hydrochoerus hydrochaeris</i> | Oct 2019      | housed in zoo          | Bennet <i>et al.</i> 2020 | T, B, B+       | MT274724.1             | MT274724.2                            |
| Yellow-necked field mouse/KS19-928/2019/Germany  | <i>Apodemus flavicollis</i>      | Sep 2019      | on zoo grounds         | Bennet <i>et al.</i> 2020 | T, B, B+       | MT274725.1             | MT274725.2                            |
| Yellow-necked field mouse/KS20-1296/2020/Germany | <i>Apodemus flavicollis</i>      | Oct 2020      | ~10 km distance to zoo | Bennet <i>et al.</i> 2020 | T, B           | MT274732.1, MT274726.1 | OL960722                              |
| Yellow-necked field mouse/KS20-1340/2020/Germany | <i>Apodemus flavicollis</i>      | 2020          | on zoo grounds         | Bennet <i>et al.</i> 2020 | T, B           | MT274733.1, MT274727.1 | OL960726                              |
| Yellow-necked field mouse/KS20-1341/2020/Germany | <i>Apodemus flavicollis</i>      | 2020          | on zoo grounds         | Bennet <i>et al.</i> 2020 | T, B           | MT274734.1, MT274728.1 | OL960725                              |
| Yellow-necked field mouse/KS20-1342/2020/Germany | <i>Apodemus flavicollis</i>      | 2020          | on zoo grounds         | Bennet <i>et al.</i> 2020 | T, B, B+       | MT274735.1, MT274729.1 | OL960724                              |
| Yellow-necked field mouse/KS20-1343/2020/Germany | <i>Apodemus flavicollis</i>      | 2020          | on zoo grounds         | Bennet <i>et al.</i> 2020 | T, B, R, P     | MT274736.1, MT274730.1 | OL960723                              |
| Yellow-necked field mouse/KS20-1512/2020/Germany | <i>Apodemus flavicollis</i>      | 2020          | on zoo grounds         | this study                | T, B, R, P     | n.a.                   | OL960720                              |
| Yellow-necked field mouse/KS20-1513/2020/Germany | <i>Apodemus flavicollis</i>      | 2020          | on zoo grounds         | this study                | T, B           | n.a.                   | OL960719                              |
| Yellow-necked field mouse/KS20-1535/2020/Germany | <i>Apodemus flavicollis</i>      | Jun 2020      | ~10 km distance to zoo | this study                | T, B           | n.a.                   | OL960718                              |
| South American coati/20_131/2020/Germany         | <i>Nasua nasua</i>               | Aug 2020      | housed in zoo          | this study                | T, B, B+       | n.a.                   | OL960717                              |
| Eurasian otter/21_002/2020/Germany               | <i>Lutra lutra</i>               | Dec 2020      | ~3 km distance to zoo  | this study                | T, B, B+       | n.a.                   | OL960716                              |

607 #T: total RNA, B: initial panRubi myBait set v1, B+ modified pabRubi myBait set v2, P: polyA selected RNA, R: rRNA depleted RNA; n.a., not applicable